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Immunophenotypic Analysis of the Transformation Zone of Human Cervix

MASSIMO RONCALLI, MARIO SIDERI, PAOLO GIÈ, AND ERNESTO SERVIDA

Departments of Pathology and of Obstetrics and Gynecology, Fatebenefratelli and Oftalmico Hospital of Milan; First Clinic of Obstetrics and Gynecology of the University of Milan, Milan, Italy

The immunocompetent cell population of the cervical trasformation zone of 18 uteri removed for noncervical disease, has been investigated with monoclonal antibodies. The panel included Leu 2a, 3a, 4, 14, and IL II receptor for lymphocytes and T cell subsets, Leu 7 for NK cells, Leu M5, Leu 10, HLA-DR, DRC 1 for dendritic cells, and Leu 6 for Langerhans' cells (LC). In ectocervical epithelium HLA-DR, Leu 6 and Leu 10 antibodies identified subpopulations of dendritic cells which differed in number and in topographic distribution. Furthermore, a strong HLA-DR epithelial positivity was constantly observed in endocervical columnar cells as well as in keratinocytes of squamous metaplasia. Leu 2a+ cells (T suppressor/cytotoxic) prevailed in the stromal and epithelial compartments of ecto/endocervix; in 6 cases, however, Leu 3a+ cells (T helper/inducer) represented the main T cell subset in the ectocervical stroma. B lymphocytes were occasionally noticed in the subepithelial stroma while NK and DRC-1 cells were never observed. Finally, only few lymphocytes displayed a positivity for IL II receptor. This study suggests that several phenotypes of intraepithelial dendritic cells are present in the transformation zone and that endocervical columnar cells and keratinocytes of squamous metaplasia express HLA-DR products; the latter finding may be related to the presence of intraepithelial and stromal T lymphocytes.

Additional key words: Cervical transformation zone, Dendritic cells, Langerhans' cells, Epithelial HLA-DR expression, Antigen presenting cells, T cell subsets, Monoclonal antibodies.

Cervical transformation zone (TZ) is the site of occurrence of viral infections and related preneoplastic or neoplastic conditions (15, 62). In general, there is increasing evidence that the development and the outcome of these pathologic events is affected by the complex relationship between offending agents and the local cellular immune response (6, 8, 37, 53, 62, 66). This latter, in turn depends on the strict cooperation between lymphocyte subsets and dendritic macrophages with antigen presenting functions (9, 10, 70). A current method of investigation of these immunologic events is based on the *in situ* identification of immunocompetent cells on normal and pathologic tissue even if a well defined immunologic function cannot always be derived from immunophenotypized cells (35). Existing immunopathologic studies of the TZ have been limited in the spectrum and specificity of antibodies employed and have been focused upon specific cell types rather than composite immunophenotypic profiles of dendritic and nondendritic mononuclear cells (34, 38, 39, 46, 48, 67, 68). In fact recent studies have investigated Langerhans' (LC) and T cell populations of the TZ either in normal or in pathologic conditions, showing a reduction of LC number in papillomavirus infection (34, 39, 67). LC are epidermotropic dendritic elements involved in antigen presen-

tation to T lymphocytes (5, 19, 55, 59, 60). In immunocompetent tissue, however, cells with supposed antigen presenting function, while showing the same dendritic morphology, display a marked topographic and phenotypic heterogeneity (19, 21, 25, 31, 32, 44, 49, 77). Furthermore, in some experimental, normal, or pathologic conditions, epithelial cells have been supposed to play an immunologic role since they express HLA-DR antigens (1–3, 30, 33, 36, 40, 47, 52, 56, 64, 65, 71, 72). These data together with the common morphologic evidence of a lympho-monocytic infiltrate in the TZ prompted us to study with a large panel of monoclonal antibodies, the immunocompetent population of this cervical site in order to characterize the lymphocytic, dendritic, and nondendritic cells of the TZ on a large homogeneous series of age-matched healthy women. This study may provide an insight into the phenotypes of cervical immunocompetent cells.

EXPERIMENTAL DESIGN

TISSUE SAMPLES

Cervical samples were collected from 18 women who underwent hysterectomy for noncervical diseases. The age ranged from 38 to 52 years. Table 1 summarizes the

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main clinicopathologic features of this series. Chronic cervicitis was always observed; in particular, in 5 cases, there was a remarkable inflammatory infiltrate of the TZ with the focal collection of nodular lymphoid aggregates containing plasma cells. In 3 cases, areas of mature squamous metaplasia were also noticed. In each case, after hysterectomy, a cervical sample of the transformation zone was immediately snap frozen in liquid nitrogen and stored at −70° C until used. For conventional immunohistochemistry, 6-μm cryostat sections were dried overnight at room temperature, subsequently fixed

TABLE 1. CLINICOPATHOLOGIC CHARACTERISTICS OF THE PATIENTS STUDIED

N	Age	Surgery	Histopathology
1	41	TAH	Severe chronic cervicitis, squamous cervical metaplasia, multiple leiomyomas, proliferative endometrium
2	42	TAH	Mild chronic cervicitis, adenomyosis, secretory endometrium
3	49	TAH, BSO	Mild chronic cervicitis, adenomyosis, proliferative endometrium
4	52	TAH, BSO	Mild chronic cervicitis, leiomyoma, secretory endometrium, bilateral hydrosalpinx, bilateral ovarian follicular cysts
5	45	TAH, BSO	Severe chronic cervicitis, multiple leiomyomas, secretory endometrium, left ovarian fibrothecoma
6	38	TAH	Mild chronic cervicitis, leiomyoma, secretory endometrium
7	47	TAH, BSO	Severe chronic cervicitis, squamous cervical metaplasia, adenomyosis, secretory endometrium, luteinized ovarian cysts
8	45	TAH	Mild chronic cervicitis, multiple leiomyomas, proliferative endometrium
9	42	TAH	Mild chronic cervicitis, multiple leiomyomas, secretory endometrium
10	48	TAH, BSO	Mild chronic cervicitis, multiple leiomyomas, secretory endometrium, left ovarian follicular cyst
11	38	TAH, RSO	Mild chronic cervicitis, uterine leiomyoma, secretory endometrium, right ovarian follicular cysts
12	47	TAH, BSO	Mild chronic cervicitis, multiple leiomyomas, proliferative endometrium, bilateral ovarian follicular cysts
13	42	TAH, LSO	Mild chronic cervicitis, multiple leiomyomas, secretory endometrium
14	42	TAH	Mild chronic cervicitis, multiple leiomyomas, proliferative endometrium
15	48	TAH, BSO	Severe chronic cervicitis, multiple leiomyomas, proliferative endometrium, bilateral ovarian follicular cysts
16	43	TAH	Mild chronic cervicitis, leiomyoma, secretory endometrium
17	39	TAH, BSO	Mild chronic cervicitis, squamous cervical metaplasia, adenomyosis, proliferative endometrium, bilateral ovarian endometriosis
18	52	TAH, BSO	Severe chronic cervicitis, multiple leiomyomas, proliferative endometrium, bilateral ovarian follicular cysts

TAH, total abdominal hysterectomy; BSO, bilateral salpingoophorectomy; RSO, right salpingoophorectomy; LSO, left salpingoophorectomy.

in absolute acetone for 10 minutes at room temperature, and air dried for 20 minutes before hydrating in phosphate-buffered saline (PBS).

IMMUNOCYTOCHEMISTRY

The monoclonal antibodies were used at the dilutions listed in Table 2. For Leu 2a, Leu 3a, Leu 4, and HLA-DR, the T cell panel for immunopathology (Becton-Dickinson, Mountain View, California) was employed, containing the primary antisera as well as the labeling reagents. All the sections were stained with the three stage monoclonal antibody avidin-biotin complex technique (76). Briefly, rehydrated sections were incubated with monoclonal antibody for 20 minutes. As second and third step labeling, the Vectastain Kit (Vector Laboratories, Burlingame, California) was employed for all but the T cell panel monoclonal antibodies. In between each step, sections were washed thoroughly in three changes of modified PBS (pH 7.4) (76). The reaction product was visualized by 3,3′-diaminobenzidine. Negative controls were obtained by omitting primary antisera, replaced by PBS; for evaluation of endogenous peroxidase activity, negative controls consisted of the use of chromogen alone.

QUANTITATION

Dendritic cells were identified by the presence of cytoplasmic processes (69) and were easily differentiated from round membrane-positive cells. Enumeration of positive cells was performed for the different phenotypes of dendritic cells (Leu 6+, Leu 10+, HLA-DR+, Leu M5+), either in the ectocervical basal and suprabasal epithelial layers or in subepithelial stroma. Total epithelial and subepithelial T lymphocytes (Leu 4) and lymphocyte subsets (Leu 2a, Leu 3a) were also evaluated.

Quantitation was performed in a controlled and reproducible way. Briefly, in each case, 3 to 6 pictures have

TABLE 2. MONOCLONAL ANTIBODIES

Antibody	Dilution	Predominant specificity
Anti-Leu 2a	Undiluted	Cytotoxic/suppressor T cells.
Anti-Leu 3a	Undiluted	Helper/inducer T cells, histiocytes (weakly).
Anti-Leu 4	Undiluted	T cells.
Anti-Leu 6	1:10	Langerhans' cells.
Anti-Leu 7	1:10	Large granular lymphocytes including NK cells.
Anti-Leu 10	1:10	Some (but not all) tissue macrophages, dendritic cells, B cells
Anti-Leu 14	Undiluted	B cells.
Anti-HLA-DR	Undiluted	Macrophages, dendritic cells, B cells, activated T cells, endothelial cells.
Anti-Leu M5	Undiluted	Monocytes, mature macrophages, dendritic cells.
Anti-DRC-1	1:10	B-zone dendritic reticular cells.
Anti-IL-11	1:10	Activated and proliferating T cells.

Sources: Anti-DRC-1 from Dako (Dakopatts, Santa Barbara, California). The remaining antibodies from Becton-Dickinson (Mountain View, California).

been taken under ×20 objective to document a representative area of the TZ on which the cell count was performed using a square grid of 6 cm². This area measured on the section 0.12 mm for each side. The mean value of positive cells counted in 6 grided areas was calculated for each case and finally expressed as number of cells/mm².

The internal consistency of quantitation technique was assessed for T lymphocytes in three compartments (ectocervical epithelium, ectocervical stroma, and endocervical stroma). The values of every case generated for each antibody (Leu 2a, Leu 3a, and Leu 4) were averaged to give the mean value for the compartment as a whole. Histiocytes which were variably Leu 3a+ were excluded from Leu 3a+ cell counts. The internal consistency was assessed by comparing the sum of Leu 2a+ and Leu 3a+ versus Leu 4+ cells in all three tissue compartments. The values in the ectocervical epithelium, ectocervical stroma, and endocervical stroma were 247 + 161 versus 383, 446 + 460 versus 929, and 505 + 270 versus 705, respectively.

RESULTS AND DISCUSSION

HLA-DR. In ectocervical mucosa, epithelial and stromal dendritic positive cells were constantly observed (Fig. 1A). Scattered subcolumnar stromal dendritic HLA-DR positive cells were also present, whereas round HLA-DR-positive mononuclear cells were only focally observed in ectocervical epithelium as well as in the stroma (Fig. 1B). Epithelial keratinocytes never expressed DR products with the exception of all three cases of squamous metaplasia, where groups of HLA-DR-positive keratinocytes were observed (Fig. 1C). Columnar cells always expressed a cytoplasmic and membranous HLA-DR positivity, strongly evident on surface epithelium and in glandular clefts (Fig. 1D and E). The membranous HLA-DR expression was seen in all aspects of plasma membranes (Fig. 1E and F). Endothelial cells of vessels were also HLA-DR-positive (Fig. 1A).

Leu 10. Dendritic Leu 10-positive cells were always found in ectocervical epithelium and in the stroma of the TZ. Intraepithelial Leu 10 dendritic positivity was observed in either the basal and the suprabasal cell layers (Fig. 1G).

Leu 6. Dendritic Leu 6-positive cells were observed in all cases only in ectocervical epithelium (Fig. 1H).

Leu M5. Round and dendritic positive stromal cells were focally identified, mainly in deep perivascular and periglandular stroma (Fig. 2). No Leu M5 intraepithelial positive cells were found.

Leu 2a, Leu 3a, and Leu 4. Positive staining of cell membranes of T lymphocytes (Leu 4) was observed in all specimens in cervical epithelium as well as in the stroma of the TZ (Fig. 2A and B). Scattered T lymphocytes were always found within the ectocervical epithelium, with the prevalence of the Leu 2a type (Fig. 2C and D). Intraepithelial endocervical T lymphocytes were also observed with a mild prevalence of the Leu 2a phenotype which constituted 60 to 70% of intraepithelial T lymphocytes (Fig. 2E). T cells subsets were mostly distributed in the subepithelial stroma (Fig. 2F and G) and some-

times clustered in nodular aggregates where Leu 3a+ cells predominated. In the TZ, some Leu 3a-positive cells with dendritic morphology were also identified.

Leu 14. Leu 14 positive cells were present only in the subepithelial stroma of the TZ, expecially in the cases showing severe chronic cervicitis.

IL II receptor. Scattered IL II receptor positive lymphocytes with a membranous pattern of staining were appreciated in the subepithelial stroma (Fig. 2H). These IL II receptor positive cells were frequently observed in the cases of severe chronic cervicitis.

DRC-1 and Leu 7. We did not observe any positive cell in the TZ when these monoclonal antibodies were tested.

CONTROLS

Controls, which consisted of the omission of the primary antisera, were invariably negative with the exception of the endoluminal content of some vessels, and of mast cells. In fact, mast cells sometimes presented a strong cytoplasmic granular reaction with avidin-biotin complex, which was easy to identify (Fig. 3A). When the chromogen alone was employed, the endogeneous peroxidase was revealed inside some vessels (Fig. 3B) and in the cytoplasm of granulocytes. Surface and glandular epithelium of the TZ resulted invariably negative (Fig. 3A and 3B). In addition, the aspecific staining was observed only in the above-mentioned cells and in vessels of the sections under study.

QUANTITATION

The comparative quantitative distribution of the HLA-DR+, Leu10+, Leu 6+, and Leu M5+ dendritic cells in the TZ is summarized in Figure 4. Dendritic cells in the basal epithelium showed a prevalence of the Leu 10 phenotype, whereas HLA-DR-positive cells were prevalent in either the suprabasal layer and the stromal compartment.

T Cell Subsets. In the ectocervical epithelium and in subcolumnar stroma, the T helper/suppressor ratio (± SE) was always less than 1, the mean values being 0.63 ± 0.06 and 0.61 ± 0.11, respectively. In ectocervical stroma, the T helper/suppressor ratio was more than 1 in 6 cases (mean 2.23 ± 0.39) and less than 1 in the remaining 12 cases (mean 0.86 ± 0.28).

DISCUSSION

Previous studies investigating the immunocompetent cell population of the TZ have focused the attention on LC (16, 34, 38, 48, 67). In our study, the phenotypic analysis of cells with dendritic morphology demonstrates that LC represent only one type of a larger and more complex dendritic cell population which expresses phenotypic and topographic heterogeneity. Quantitative study indicates that most of the dendritic cells are HLA-DR+ while Leu 10+ dendritic cells represent a smaller population. Anti-Leu 10 recognizes an Ia determinant, HLA-DC/DS, distinct from HLA-DR, which has been correlated with an enhanced antigen presenting capability (22). Alternatively, Most, Knapp, and Wick (40) have suggested that HLA-DC/DS can act as immune suppression gene, possibly by activating T suppressor cells. This
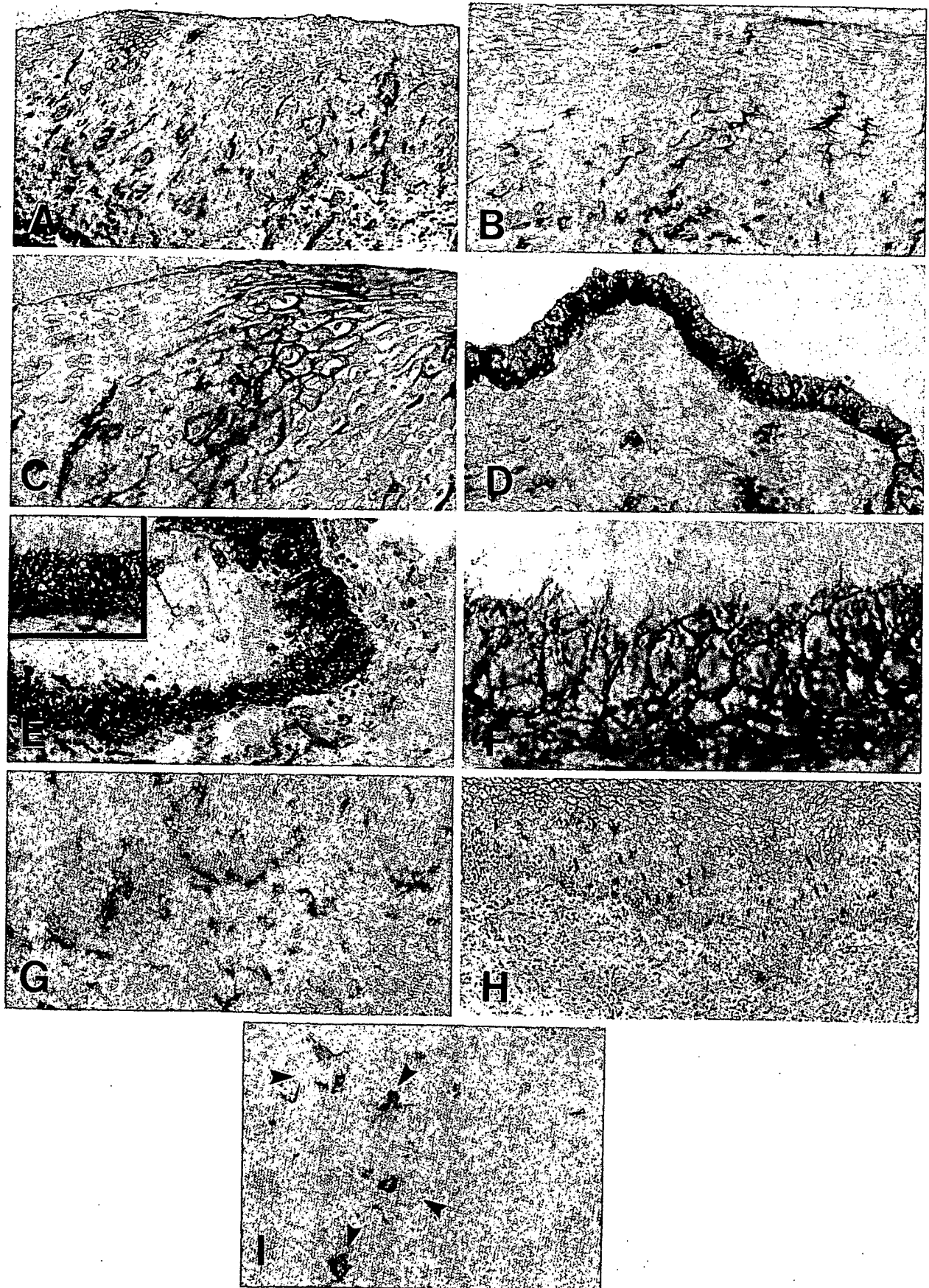
FIG. 1. Pattern of HLA-DR, Leu 10, Leu 6, and Leu M5 positive cells. A to F, HLA-DR. A, Dendritic intraepithelial and stromal positive cells are clearly evident, (*bottom left*), a glandular endocervical cleft lined by HLA-DR+ columnar cells and some HLA-DR+ vessels (×100, nuclear counterstain). B, Dendritic and round positive cells in ectocervical mucosa (×250, nuclear counterstain). C, An area of squamous metaplasia with keratinocytes showing marginally located cytoplasmic and surface HLA-DR positivity (×250, nuclear counterstain). D, Sur-
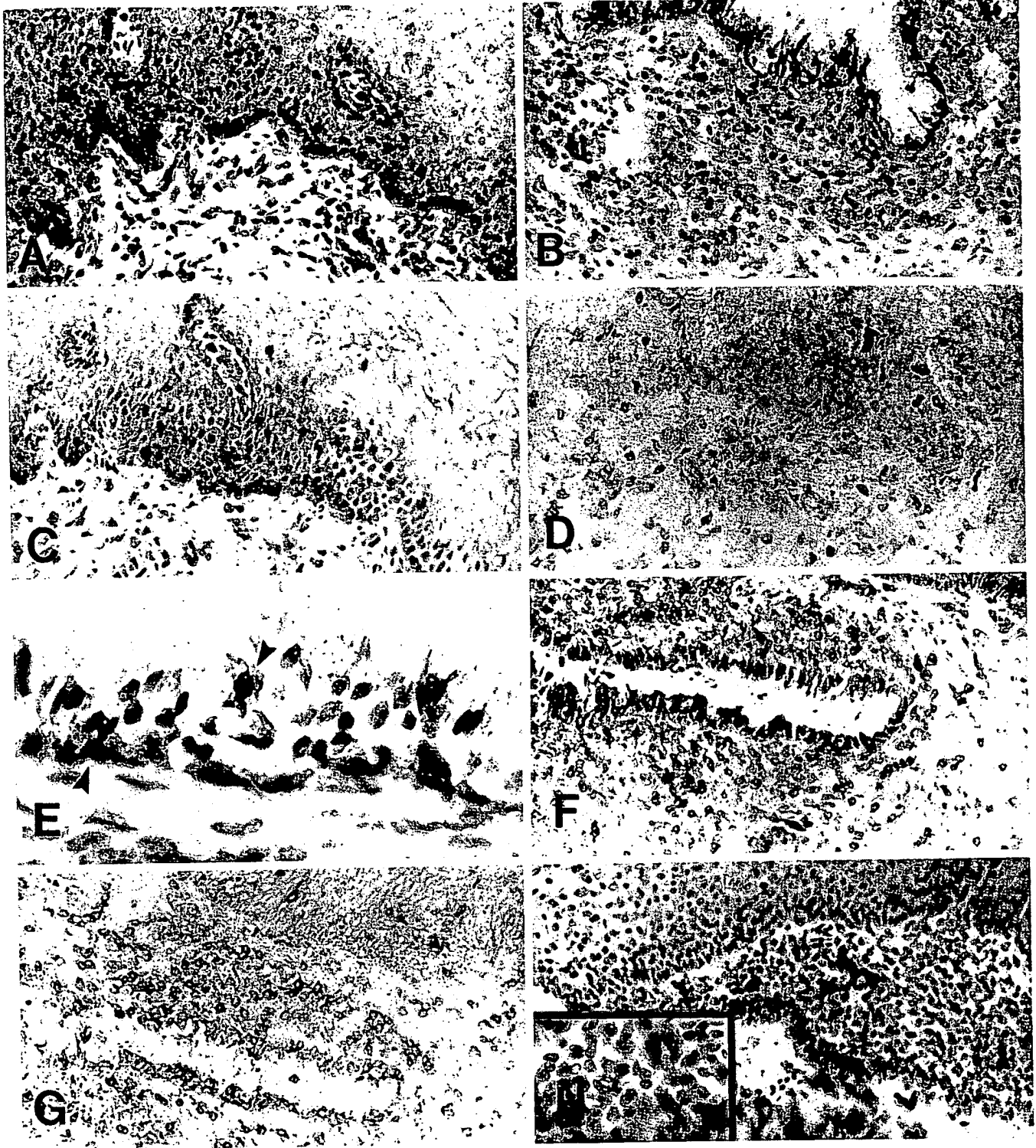
FIG. 2. Pattern of Leu 2a, Leu 3a, Leu 4 and IL II receptor positive lymphocytes. *A* and *B*, Leu 4. T lymphocytes are clearly evident either in ectocervical mucosa (A, × 250, nuclear counterstain) or in endocervical stroma (*B*, × 250, nuclear counterstain). *C* to *G*, Leu 2a and Leu 3a. *C*, Intraepithelial T lymphocytes are mainly of the Leu 2a type (×250, nuclear counterstain) as demonstrated by the comparison with *D*, showing the same field stained with Leu 3a (×250, not counterstained). *E*, Intraepithelial Leu 2a+ cells (*arrows*) in the endocervical mucosa (×400, nuclear counterstain). *F* and *G*, Same field of endocervical stroma respectively stained with Leu 2a and Leu 3a: stromal T lymphocytes are either of the T helper or T suppressor type (×250, Leu 2a with nuclear counterstain; Leu 3a, not counterstained). *H*, IL II receptor. Scattered positive stromal lymphocytes (×250, nuclear counterstain). *Inset*, Note the characteristic IL II receptor membranous pattern of positivity (×400).

face HLA-DR positive columnar cells (×300, nuclear counterstain). *E*, The same pattern of positivity of surface endocervical cells is observed in columnar cleft epithelium (×250, nuclear counterstain). *Inset*: Cytoplasmic and membranous HLA-DR positivity (×400). *F*, At higher magnification, HLA-DR details the cytoplasmic membrane of columnar cells (×1,000). *G*, Leu 10. Intraepithelial and stromal dendritic-positive cells. Note positive dendritic intraepithelial cells rimming the basal cell layer (×250, not counterstained). *H*, Leu 6. Langerhans' cells in the suprabasal ectocervical epithelium. No Leu 6-positive cells are present in the stroma (×100, nuclear counterstain). *I*, Leu M5. Leu M5 reveals few macrophages (*arrows*) in the subepithelial stroma (×400, not counterstained).

latter hypothesis fits well our observation of T suppressor/cytotoxic cells as the main lymphocytic population in the ectocervical epithelium of the TZ.

A third dendritic cell population, corresponding to LC, expresses the Leu-6 phenotype (17, 41–43). LC are identified by the ultrastructural evidence of the characteristic Birbeck's granule (4). In cervical tissue, previous investigators employed a spectrum of different antisera with variable specificity to identify LC (34, 38, 39, 48, 67). However, at present, LC are commonly immunohistochemically characterized by the Leu 6+/HLA-DR+ phenotype (11, 20, 27, 51, 73) and we have referred to this phenotype to define LC. Our quantitative results are in agreement with the study of Morris et al. (38), who found, on a smaller series, that the average number of LC/mm^2 varied from 74 to 145. However, we did not observe "hugging" cells located across the basement membrane. In fact, we found that LC are only present within the epithelium and, in particular, in the suprabasal layers. Our quantitative analysis indicates that, in suprabasal epithelial layers, HLA-DR+ dendritic cells outnumber Leu 6+ cells. We do not know the exact nature of these
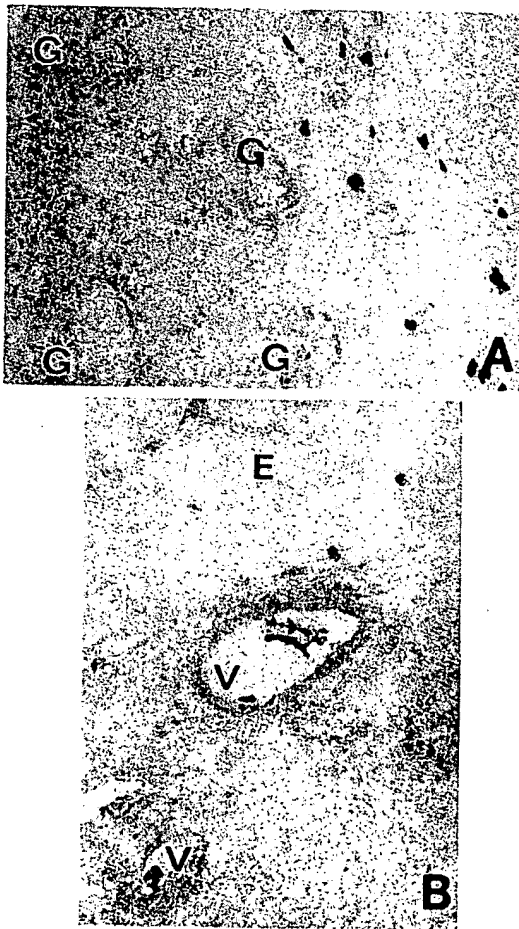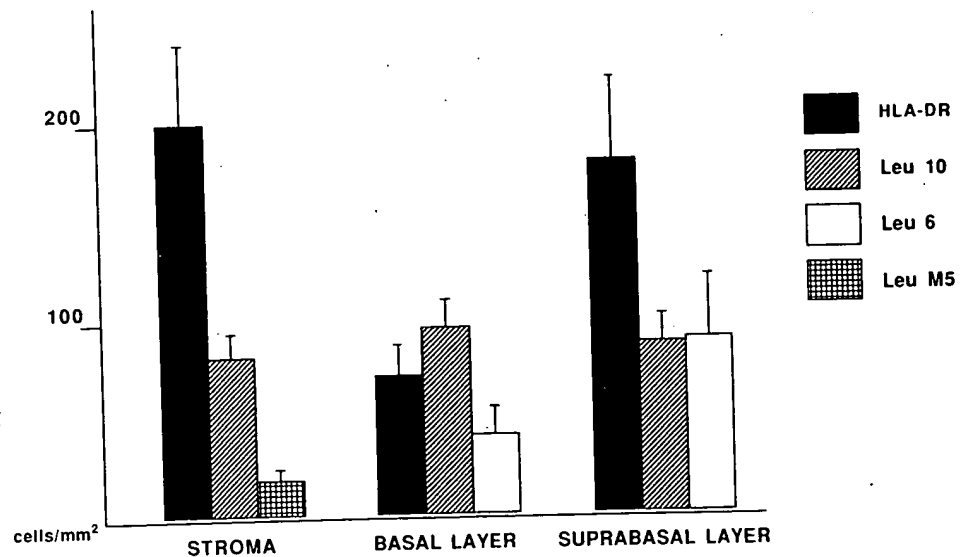

FIG. 3. Control cases. A, When primary antiserum was replaced by PBS, only some mast cells stained with ABC. Notice the unstained glandular clefts (G) (×100, nuclear counterstain). B, When the chromogen alone was employed, the endogenous peroxidase was revealed inside some vessels (V). Notice the unstained ectocervical epithelium. (×250, not counterstained).

cells, but relying on their dendritic features, and their immunophenotypes (HLA-DR+/Leu 10−/M5−/ Leu 6−) we can speculate that these cells belong to the group of dendritic antigen presenting cells (21, 29, 32, 77). Finally, scattered dendritic Leu M5+ cells were distributed in the subepithelial stroma of ecto-endocervix. These cells are a minor population, which probably represents mature resting macrophages. Our study suggest the presence of different phenotypes of dendritic intraepithelial cells in the TZ. The phenotype of these dendritic cells is consistent with an antigen presenting function; however, a clear distinction among these cells, based only on immunohistochemical staining is not feasible and further studies are needed to clarify this issue.

Ia-like antigen expression by epithelial cells has been demonstrated in a wide variety of normal and abnormal conditions (2, 3, 18, 23, 26, 33, 36, 45, 56, 61, 64, 75). In particular in the cervix Morris et al. (38) and Puts et al. (48) have reported a focal HLA-DR positivity of endocervical cells. In our study, a strong epithelial HLA-DR positivity was constantly observed in columnar cells. Since chronic cervicitis with intraepithelial T lymphocytes was present in all the cases, this epithelial HLA-DR expression may be related to the abundance of T cells. In fact, in some experimental and pathologic conditions, the HLA-DR expression is induced by activated T lymphocytes as clearly demonstrated in autoimmune thyroid disease (7). The remarkable inflammatory infiltrate observed in our cases, near areas of squamous metaplasia with HLA-DR+ keratinocytes is in further support of this suggestion. In addition, a strong epithelial HLA-DR expression has been recently reported in human endometrium adjacent to lymphoid aggregates or areas of chronic endometritis by Tabibzadeh et al. (64). Furthermore, the same authors correlated the epithelial HLA-DR expression with the hormonal cycle, whereas in our study, the endocervical HLA-DR positivity was similarly expressed either in proliferative or in secretory phases. This is probably because the cervical epithelium does not undergo the sequences of proliferation-differentiation-secretion and shedding as seen in endometrium.

As to the immunologic significance of HLA-DR positivity on epithelial cells, Unanue and Allen (71) speculate that this antigenic expression may be related to an antigen presenting capability, although other immunologic or nonimmunologic roles cannot be excluded. According to this hypothesis, an antigen presenting capability of endocervical columnar cells is consistent with the lack of endocervical dendritic cells which, in turn, are well represented in the pluristratified squamous counterpart of the TZ.

The study of lymphocyte populations in the TZ shows that T lymphocytes greatly outnumber B lymphocytes. This finding is in keeping with the widespread presence of antigen presenting cells, which are thought to have T lymphocytes as targets (10, 50, 70, 74). As reported by other authors (38, 46, 68), T suppressor/cytotoxic cells are the main lymphocyte subset in either epithelial or stromal compartments. In particular, in ectocervical epithelium Ts cells are in close relationship with both LC

FIG. 4. Quantitative topographic comparison of HLA-DR, Leu 10, Leu 6, and Leu M5 positive dendritic cells in the epithelium and subepithelial stroma of the ectocervix. The figure represents the general averaged values of the different phenotypes of dendritic cells (mean ± SE).

and HLA-DR+ dendritic cells. This finding is in keeping with the demonstration that LC are required for the generation of cytotoxic T lymphocytes (11, 13, 59); furthermore, the response of cytotoxic T lymphocytes to allogenic targets is greatly enhanced when a class II major histocompatibility antigenic stimulus is also provided (14, 28, 54, 57, 58). In the subepithelial ectocervical stroma, however, one-third of the cases showed a prevalence of Leu 3a+ cells, especially in the cases showing B lymphocytes and plasma cells. The latter observation, as suggested by Morris et al. (38), may be correlated with the known role of T helper/inducer cells in differentiating B lymphocytes into plasma cells. IL II receptor positivity, indicative of functionally active and proliferating lymphocytes (24), was focally expressed, especially in cases of severe chronic cervicitis. This finding confirms the resting condition of most of the lymphocytes present in the human adult cervical mucosa, and correlates well with the almost total absence of NK cells. This latter observation, however, is in disagreement with that reported by Syrjanen et al. (63), who found scattered NK cells in the cervical TZ. The clinical characteristics of the population studied by these authors, mainly affected by HPV infection, may account for these discrepancies.

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